A RENAISSANCE OF CYTOGENETICS: STUDIES IN POLYPLOIDY AND CHROMOSOMAL EVOLUTION[1]

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ABSTRACT

Systematics and evolutionary biology are being bolstered by a renaissance in cytogenetics and comparative genomics as illustrated by reviewing Peter Raven's integration of cytogenetics and phylogenetics and by presenting updates to his work in three key research areas. The first area is the evolution of chromosomes during the origin of the angiosperms. Raven's analysis of chromosome numbers in the Annonales and other basal angiosperms inspired modern genomic comparisons that have revealed paleopolyploid events, which appear to have occurred early and often during flowering plant diversification. Second, Raven's characterization of chromosome evolution in various genera of Onagraceae is updated in light of a contemporary Onagraceae phylogeny. The possible construction of ancestral karyotypes in the Onagraceae is feasible using techniques that have been successful in analyses of genomic blocks in the Poaceae and Brassicaceae. Third, Raven's work on catastrophic speciation identified the importance of chromosomal rearrangements in the evolution of *Clarkia* Pursh and the ability of new species to inhabit different environments. Current work in *Brassica* L. has shown that phenotypic changes contributing to speciation events can arise from relatively few chromosomal rearrangements. A fusion of systematics and cytogenetics is opening new areas of research, with phylogenomics allowing ancestral genome reconstruction, the incorporation of genome-level characters into phylogenetic analyses, and new theories about evolution on a genomic scale.

Key words: *Brassica*, Brassicaceae, chromosome evolution, *Clarkia*, molecular cytogenetics, Onagraceae, phylogenetics, phylogenomics, Poaceae, polyploidy.

In 1986, Peter Raven commented, "a genuine conceptual difficulty with the field of biosystematics is that it was always conceived as a way of improving plant classification, rather than a way of learning more about plant evolution" (Raven, 1986: 26). Indeed, the history of biosystematics itself is problematic. The origin of phylogenetics in the mid-20th century caused drastic changes in how systematics is viewed philosophically. Before the advent of phylogenetics in the mid-20th century, biosystematists had concentrated on closely related species and within-species variation, with methodologically and theoretically flexible views of breeding and chromosomes, viewing evolutionary relationships as webs. The arrival of tree thinking switched the focus of systematics primarily toward resolving phylogenetic relationships of more distantly related species and higher taxa, with a more monistic interpretation of traits and homology (Sytsma & Pires, 2001). In 2006, Peter Raven remarked in personal communication, "the two biggest advances in my lifetime were (1) the conceptual distinction between synapomorphy and pleisiomorphy and (2) the development of technology to allow us to look at chromosomes, then flavonoids, then isozymes, and finally DNA." The most important advancements over the past several decades were improved methods of building trees and visualizing phylogenetic relationships. While the importance of phylogenetics need not be questioned, we can ask whether its growth comes at the expense of other disciplines, such as evolutionary development and cytogenetics. The past few decades have shown how a field such as evolutionary development (evo-devo) can emerge, suggesting the potential for resurgence of other fields (Love, 2003; Breidbach & Ghiselin, 2007; Muller, 2007; Pigliucci, 2007).

Here, we argue that a similar renaissance in cytogenetics and comparative genomics is occurring

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with the advent of modern cytogenetic techniques and methods for analyzing genomes. We illustrate this by reviewing Peter Raven's integration of cytogenetics and phylogenetics and by presenting updates to his pivotal work in three key research areas. First, how can the chromosome numbers of basal angiosperms contribute any information to chromosome evolution across angiosperms? Raven's investigation of chromosome numbers in the Annonales supports subsequent conclusions about the basal chromosome number of angiosperms, and these analyses inspired genomic comparisons that revealed paleopolyploid events. Second, how have chromosomes evolved in Onagraceae? Raven's characterization of the types of chromosomes found in various genera of Onagraceae reveals interesting patterns of chromosomal evolution when mapped onto the contemporary Onagraceae phylogeny. Recent advances in similar work allow the construction of ancestral karyotypes in grasses and analyses of genomic blocks in Brassicaceae. Finally, how have chromosomal rearrangements led to rapid speciation in the genus *Clarkia* Pursh? Work in catastrophic speciation, which occurs when a minority cytotype becomes adaptively advantageous following dramatic habitat change, allowed Raven to identify the importance of chromosomal rearrangements in *Clarkia* speciation, and new examples from the same genus highlight the abilities of these species to inhabit different environments. Current work in *Brassica* L. has consequently shown that relatively few chromosomal rearrangements can lead to phenotypic changes that may contribute to speciation events.

BASAL CHROMOSOME NUMBER IN THE ANGIOSPERMS

From a phylogenetic viewpoint, the term basal is used to denote organisms that are placed at or near the base of a phylogenetic tree, and is always used in reference to a certain group of organisms. When discussing deep phylogenies of angiosperms, authors often refer to groups of organisms whose branches originate near the base of the tree, such as *Amborella* Baill. and the Magnoliales, as basal angiosperms. In fact, these plants are merely descendants of the true ancestral angiosperms, which are presumably now extinct (Crisp & Cook, 2005). Indeed, *Amborella* and all extant flowering plant lineages are equally distant from a common ancestor. For the purposes of this paper and in concordance with the literature being reviewed, the lineages of plants that diverged from a common angiosperm ancestor prior to the monocot-eudicot split will be referred to as basal angiosperms, with the understanding that collectively they are merely extant approximations of the true ancestors of flowering plants.

One of Raven's earliest contributions to cytology involved the basal chromosome number in the angiosperms (Raven & Kyhos, 1965), and these conclusions have been updated by more recent chromosome counts (Soltis et al., 2003). The advent of genome sequencing has consequently allowed comparisons between species for more thorough analyses of chromosomal evolution. It was Raven's thought that chromosome counts from the Annonales, or the woody Ranales, might shed light on angiosperm base chromosome number. His 1965 paper published the chromosome counts for *Degeneria* I. W. Bailey & A. C. Sm. ($n = 12$) and *Drimys* J. R. Forst. & G. Forst. (43 bivalents). These counts led Raven and Kyhos (1965: 247) to state, "with our present information, it appears very likely that $n = 7$, as suggested by Darlington (1956) is the original base chromosome number of angiosperms." This conclusion seemed fairly reasonable; a relatively low base chromosome number is selectively advantageous because it reduces recombination (Raven & Kyhos, 1965: 247). However, Raven cautioned that aneuploidy and polyploidy confound the issue, and phylogenetic considerations should be applied to interpret the base chromosome number of angiosperms. Moreover, "there are still six families of annonalean affinities for which not a single chromosome count is available" (Raven & Kyhos, 1965: 247). Raven's interest in comprehensive chromosome counting did not stop with this paper, however; he was instrumental to the founding of the Index to Plant Chromosome Numbers (IPCN Series), which originated at the University of California, Berkeley, with Marion Cave's efforts in 1956, but moved to the Missouri Botanical Garden in 1978.

Subsequent research in major patterns of cytology and phylogeny in the angiosperms has confirmed the relevance of Raven's conclusions regarding basal chromosome numbers in angiosperms. Major developments facilitating current advancement include a more accurate phylogeny of angiosperms and more chromosome counts from taxa of early diverging lineages. Subsequent researchers have continued to fill in gaps in chromosome numbers to provide a more complete picture of chromosome evolution in angiosperms (Ehrendorfer et al., 1968; Walker, 1972; Ehrendorfer & Lambrou, 2000). Additionally, we are gaining a better understanding of relationships among the basal angiosperms; current phylogenies place *Amborella* and *Nuphar* Sm. as sister groups to the rest of the flowering plants. Paradoxically, many basal angiosperms are now known to have high haploid chromosome numbers (i.e., *Amborella* with $n = 13$ [Oginuma et al., 2000], *Illicium* L. with $n = 11$ [Fedorov, 1969], Magnoliaceae with $n = 19$ [Fedorov, 1969]). Despite these observations, current thought indicates the original base chromosome number is low ($x = 6$–9) (Raven, 1975; Soltis et al., 2003, 2005).

Current methods of characterizing genome evolution go beyond mapping chromosomes onto phylogenies by exploring the prevalence of polyploidy and other genome duplications in angiosperms. A variety of research methods, including isozymes and phylogenomics, suggest that genome duplications have arisen in many angiosperm families, with repeated cycles of polyploidization and diploidization. Classic estimates of genome evolution in polyploidy indicate 30%–50% of extant angiosperms are polyploids, and recent studies suggest that most extant angiosperms share at least one polyploid ancestor (Cui et al., 2006). Isozymes first detected duplicated loci in Magnoliaceae and other basal lineages, providing the first suggestion of paleopolyploid events; isozyme evidence for duplications in eudicots confirmed earlier hypotheses of polyploidy in angiosperms (Soltis, 2005).

A variety of inventive methods of genome analysis have been applied to detect the history of whole-genome duplications in angiosperms. Bowers (2003) fused phylogenetics and genomics to evaluate ancient genome duplications within *Arabidopsis* (DC.) Heynh. and found three nested polyploid events. The ages of duplicated genes suggest an alpha duplication event after the Brassicaceae-Malvaceae split, an older beta duplication event after the monocot-eudicot split, and perhaps a more ancient gamma duplication event that is difficult to place. Comparisons of sequenced genomes reveal duplicated genes, which provide evidence of ancient polyploidy in *Arabidopsis*, *Oryza* L., and *Populus* L. (Blanc et al., 2003; Paterson et al., 2004; Yu et al., 2005). In a parallel effort, Floral Genome Project investigators examined basal angiosperm lineages for older polyploidy events that predated the monocot-eudicot divergence. Ks analyses revealed independent ancient polyploidy events in several early diverging angiosperm lineages. Much slower rates of sequence evolution occur in basal angiosperms, especially *Liriodendron* L. (Magnoliaceae), allowing analyses to determine that at least one ancient polyploidy event occurred in the common ancestor of all angiosperms or in the common ancestor of all angiosperms other than *Amborella* (Cui et al., 2006).

Raven's early work counting chromosomes in the Annonales allowed him to make hypotheses about the base chromosome number for all angiosperms. This type of work laid the foundation for more thorough analyses of chromosome number with the discovery of other basal angiosperm lineages, which, in turn, have advanced more in-depth investigations of chromosomal evolution and ancestral karyotype inferences via phylogenomic comparisons.

CHROMOSOME EVOLUTION IN ONAGRACEAE

Although Peter Raven's ideas about basal angiosperm chromosome number were insightful, he is perhaps more famous for his work on chromosome evolution within the Onagraceae, the evening primrose family. His initial characterization of Onagraceae chromosomes eventually led to hypotheses about the phylogenetic patterns in the family, and current work on ancestral karyotype reconstruction in the Poaceae and Brassicaceae reaffirms the importance of addressing chromosomal organization in a phylogenetic framework.

An early collaborative work on mitosis in the Onagraceae covered all tribes, 15 genera, and 46 species. This comparative work described three modally distinct groups of chromosomes that vary in size and heterochromatic content. Members of the first group of chromosomes are presumably ancestral and medium sized, contract evenly along length during meiosis, and are not visible in interphase; they are found in *Fuchsia* L., *Circaea* L., and *Lopezia* Cav. The second group is intermediate in age; these chromosomes are morphologically dot-like and heteropycnotic throughout meiosis, and are not visible in interphase; they are found in *Ludwigia* L., *Epilobium* L., and *Boisduvalia* Spach. Chromosomes in the third group are derived, medium sized, and heteropycnotic near the centromere, and have long, lightly staining euchromatic tails; they are found in *Hauya* DC. and *Onagreae*. These groupings were used to support *Fuchsia* and *Ludwigia* as less-derived genera of the family (Kurabayashi et al., 1962).

When combined with Raven's classification of Onagraceae chromosomes, the current Onagraceae phylogeny (Levin et al., 2003) suggests subtle patterns about chromosome evolution. The previously mentioned chromosome types (Kurabayashi et al., 1962) do not map simply onto the Onagraceae phylogeny; small, dot-like chromosomes have evolved independently multiple times. Later karyomorphological studies in the Onagraceae described four types of chromosomes (Tanaka et al., 1988). There were two major differences between the old and new chromosome groupings. In the new chromosome classification scheme, the genera of section *Onagreae* were split into two groups, and both the first and second groups in the original classification were reorganized. This new chromosome classification scheme suggests variation in the origin of chromosome morphology and indicates that cytology of genera reflects phylogenetic relationships. For example, the independent evolution of small chromosomes in Onagraceae can be explained by more thorough analyses of chromosome structure. Although *Epilobium* and *Ludwigia* both have small chromosomes, *Epilobium* chromosomes seem to be a specialization of more diffuse (type 1) chromosomes (P. Raven, pers. comm.).

Recent comparative genomic analyses of the Poaceae and Brassicaceae are illustrative of potential future practices in family-wide plant systematic studies. Comparisons among grass genomes indicate that there is colinearity among the grass genomes with rice as the central reference genome (Devos et al., 2002). The benefits of this comparative approach (e.g., using synteny to find candidate genes in non-model taxa) make it a desirable analytical tool to apply to other taxonomic groups with copious genetic information available. For example, is it possible to compare colinearity among crucifer genomes with *Arabidopsis thaliana* (L.) Heynh. as a reference? Although *A. thaliana* will continue to be the functional reference point, its small, derived genome ($n = 5$) complicates larger-scale structural genomic comparisons to other members of the Brassicaceae. Given that complete genome sequences for at least four crucifers (*A. lyrata* (L.) O'Kane & Al-Shehbaz [$n = 8$], *Capsella* Medik. [$n = 8$], *Brassica rapa* L. [$n = 10$], and *Thellungiella* O. E. Schulz [$n = 7$]) will be available within a few years, it is unlikely that *A. thaliana* alone will continue to be used for structural comparisons. It may be possible for genetic maps and cytogenetic data from several Brassicaceae genomes to be combined to create a generalized reference set of ancestral genomic blocks. The *A. thaliana* genome can be split into at least 21 conserved blocks, and comparative analyses reveal *A. thaliana* and *B. napus* L. share large, colinear blocks that have undergone numerous rearrangements (Parkin et al., 2005). These types of structural comparisons allow extrapolations about the evolution of more derived states. In fact, the karyotype of *A. thaliana* is derived from a tentative $n = 8$ ancestral karyotype; genetic maps of closely related species, *A. lyrata* and *C. rubella* Reut., are collinear ($n = 8$) and show how *A. thaliana* ($n = 5$) is rearranged and derived (Lysak et al., 2006). Investigating the *A. thaliana* and *Brassica* genomic blocks with other crucifer genomes will allow for the reconstruction of an ancestral karyotype, which will better facilitate comparisons across the Brassicaceae (Schranz et al., 2006).

Peter Raven's analysis of chromosomal forms in Onagraceae provided invaluable information to researchers who could later analyze chromosomal evolution in the family. Current research in Poaceae and Brassicaceae has built on similar types of analyses by describing how genomic blocks can show conserved and derived patterns of chromosomal evolution across closely related species. These studies indicate a major role for cytogenetic data in phylogenomic comparisons. At the family level, analyses of chromosomal blocks and ancestral karyotype reconstruction will most likely be developed first in Poaceae, Brassicaceae, Leguminosae, Solanaceae, and Asteraceae. However, as genomic resources and comparative analyses improve, Onagraceae, and any other family of choice, will not be far behind. One can imagine the possibility of generating ancestral genome browsers for deeper-nested nodes (e.g., Brassicales, rosids, eudicots, angiosperms) that would allow for a myriad of phylogenetic inferences and comparisons across clades of flowering plants.

RAPID SPECIATION IN THE GENUS *CLARKIA* AND CHROMOSOMAL REARRANGEMENTS

Peter Raven's research interests not only spanned comparisons of orders and families of flowering plants but also involved integrating phylogenetics and cytogenetics within genera and species. Specifically, Raven's theories about catastrophic selection and edaphic endemism as a factor in speciation later informed phylogenies about *Clarkia* and modes of adaptation to habitats. Modern research incorporating cytogenetics and phenotype change in *Brassica* confirms the ability of species to adapt quickly to new environments and to become reproductively isolated.

Early work by Lewis (1962) theorized that the removal of most of a population would allow a deviant, or better adapted, genome to establish a new marginal population. This concept of catastrophic speciation was later expounded upon by Raven (1964: 338) when he suggested that "catastrophic selection and consequent genotype reorganization may be a potent force in the creation of new edaphically restricted endemics." He noted a pattern possibly supporting this hypothesis in *Clarkia rubicunda* (Lindl.) F. H. Lewis & M. E. Lewis and *C. franciscana* F. H. Lewis & P. H. Raven, but found further support in distinctive endemics like *Microcycas* (Miq.) A. DC. (Mason, 1946) and narrow endemics like the *Streptanthus glandulosus* Hook. complex (Kruckeberg, 1957).

Recent phylogenetic and cytogenetic analyses of *Clarkia* reveal fascinating patterns of speciation and chromosomal evolution. Although very closely related with little sequence divergence, *Clarkia biloba* A. Nelson & J. F. Macbr. ($n = 8$) and *C. lingulata* F. H. Lewis & M. E. Lewis ($n = 9$) are effectively reproductively isolated due to hybrid sterility; their differences in base chromosome numbers result in poor chromosome pairing (Sytsma & Smith, 1992). In contrast, *Clarkia* sect. *Rhodanthos* subsect. *Rhodanthos* presents a more complicated example. Several researchers have utilized different types of data to determine the mode of speciation in this group.

Subsequent models of speciation project modes of speciation at different points in time (Lewis & Raven, 1958; Gottlieb, 1973; Sytsma et al., 1990; Gottlieb & Edwards, 1992). *Clarkia*, therefore, provides an interesting and complex system in which to examine chromosomal speciation, as it involves several modes of chromosomal evolution: shifts in base chromosome number, genome downsizing, and polyploid speciation.

While work on *Clarkia* has revealed the formation and establishment of taxa through catastrophic speciation, newer genomic methods are beginning to sort out mechanisms through which this process can occur. Work in diploid *Helianthus* L. hybrids (Rieseberg et al., 2003) and allopolyploid *Brassica* (Pires et al., 2004; Gaeta et al., 2007) suggests molecular mechanisms that can generate rapid variation, which may support the ecological theory of catastrophic speciation. A modern application of Peter Raven's work on speciation in *Clarkia* is found in analyses of resynthesized *Brassica* allopolyploids, which show rapid chromosomal and phenotypic change. In brief, chromosomal rearrangements caused by homoeologous recombination can alter the dosage of homoeologous alleles. This variable dosage of alleles can result in novel variation for flowering time, and genotypes with rapidly diverging flowering times can become isolated in the absence of cross pollination of early and late flowering groups. These studies of resynthesized *B. napus* polyploids elucidate potential mechanisms of speciation because rapid changes in genome structure alter gene expression and result in phenotypic variation of important life history traits like flowering time (Pires et al., 2004; Gaeta et al., 2007).

Initial work by Peter Raven on catastrophic selection in *Clarkia* suggested that dramatic changes in phenotype could arise from chromosomal changes and become fixed in a population; later work in *Clarkia* reaffirmed this pattern throughout the genus. Current research in taxa like *Helianthus* and *Brassica* also reveals that chromosomal rearrangements can cause phenotypic change that may eventually lead to speciation.

TOWARD A CYTOGENETIC RENAISSANCE IN SYSTEMATICS

Peter Raven left his mark on several areas of research that have been continually reexamined and expanded. His ideas regarding the basal chromosome number in angiosperms were eventually confirmed by updated phylogenies and corresponding chromosome counts of newly identified basal angiosperm lineages. In turn, these studies have led to genome-wide investigations that have detected signatures of ancient polyploidy events occurring throughout the diversification of the angiosperms. Raven's characterization of chromosome types in Onagraceae has revealed complex evolutionary patterns in the family; these types of analyses have been extended in grasses and Brassicaceae to show how chromosomal blocks can be compared between organisms. Raven's work on catastrophic selection in *Clarkia* provided the theoretical framework for further research on the molecular mechanisms of chromosomal rearrangements and rapid speciation.

The revolution in evolutionary development was made possible by the formation of new, accessible methods of experimentation and analysis. Similarly, the cytogenetic renaissance will depend upon new methods that facilitate combining cytogenetic and phylogenetic analyses. The key to ushering forward the cytogenetic renaissance will arise from conceptually linking cytogenetics and phylogenetics by utilizing new cytogenetic tools, improving sequencing technologies, and cultivating phylogenomics (including comparative chromosomics) as a research priority (Dobigny et al., 2004; Graphodatsky, 2007).

First, new cytogenetic tools and applications will make cytogenetic research more accessible. Repetitive DNA sequences, like retroelements, are very useful for easily labeling chromosomes from parental species in a hybrid. Fluorescent in situ hybridization (FISH) probes for retroelements, as well as many other types of probes, can be quickly obtained from a variety of library-building techniques. One relatively inexpensive and efficient protocol for building small insert libraries (about 1000 bp) produced probes that effectively labeled retroelements from the genera *Zea* L. and *Tripsacum* L. (Lamb & Birchler, 2006). New techniques in FISH are allowing easy visualization of gene clusters or even single genes (Kato et al., 2006); regions as small as 3 kb can be effectively probed with these methods. These new developments are making cytogenetics accessible and useful for anyone interested in chromosomal evolution.

Second, new sequencing technology will alter the availability of data for phylogenomic analyses. Genomic sequences for select species are allowing more rapid development of nuclear gene loci that could potentially be used for phylogenetic analyses (Small et al., 2004; Gilchrist et al., 2006; Bouck & Vision, 2007). Complete genome sequences can be found in nearly every plant group (gymnosperms, moss, fern, fungi, algae), allowing all plant biologists to take advantage of this information. The use of genomic data for systematics will initially derive from mining complete genome sequences from closely related crop and model species, but the advent of low-cost genome sequencing technology and phylogenetically informed genomic comparisons (e.g., Rokas et al., 2003; Sjolander, 2004; Delsuc et al., 2005; Koonin, 2005; Philippe et al., 2005; Jeffroy et al., 2006; Jansen et al., 2007; Philippe & Blanchette, 2007) will provide resources for many species in question.

Finally, the cultivation of phylogenomics as a research area will actively integrate cytogenetics and genomics with phylogenetics. Phylogenomic analyses will ultimately establish not only evolutionary relationships among organisms but also relationships among all genes within an organism. A phylome, the complete collection of all gene phylogenies in a genome, has been developed for humans (Huerta-Cepas et al., 2007) and in the future will be developed for plants. Currently available resources such as bacterial artificial chromosome (BAC) libraries and expressed sequenced tag (EST) libraries can both be used by systematists to assess gene copy number and integrate cytogenetic data and nuclear genes with phylogenies. For example, Pires et al. (2006) utilized genome size and chromosome number to explore genome evolution in Asparagales and proposed that far more information could be unearthed using BACs or ESTs from crop plants like asparagus and onion to investigate non-model taxa like hyacinths, agave, amaryllids, irises, and orchids. The proliferation of genomic resources coupled to a fusion of systematic and cytogenetic methodologies promises to open up whole new areas of research. Phylogenomics will allow for ancestral genome reconstruction, the incorporation of genome-level characters into phylogenetic analyses, and new theories about evolution on a genomic scale.

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